Efficacy of *Beauveria bassiana* for control of *Tribolium castaneum* with reduced oxygen and increased carbon dioxide

J. C. Lord

USDA-ARS, GMPRC, Manhattan, KS, USA

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Correpondence
Jeffrey C. Lord (corresponding author), USDA-ARS, GMPRC, 1515 College Avenue, Manhattan, KS 66502, USA.
E-mail: jeff.lord@ars.usda.gov

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Abstract

This study investigated the effect of atmosphere modification, a widely adopted means of insect control in stored products, on the efficacy of *Beauveria bassiana* for one of the most difficult to control pests, *Tribolium castaneum*. Oxygen reduction to 5% (±1%) as opposed to CO_2 elevation to 40% (±2%) for the first 72 h of fungus exposure resulted in significantly greater larval mortality than fungus exposure under ambient atmospheres. Both treatments reduced pupation of older larvae suggesting that slowed development may be a beneficial factor for fungal efficacy. CO_2 elevation but not O_2 reduction significantly affected the mortality of adult beetles that were exposed to the fungus. Carbon dioxide elevation significantly reduced *B. bassiana*'s germination and growth rates, but oxygen reduction did not.

Introduction

Atmosphere modification by reducing oxygen or increasing carbon dioxide has been used widely in recent years as a chemical-free strategy for managing stored-product pest insects (Donahaye et al. 1996; Adler et al. 2000; Jayas and Jeyamkondan 2002). The red flour beetle, *Tribolium castaneum* (Herbst), being among the most problematic of stored-product insects, has been the subject of considerable research on the approach (Press and Harein 1967a; Storey 1977). This can be done by introducing gases or by hermetically sealing storage units. An approach proposed by Conyers and Bell (2007) is to employ moderate O_2 reduction and CO_2 elevation by continuous input of gases as might be accomplished with a gas burner. The authors expect this to cause life cycle disruption rather than complete mortality. Regardless of the approach, treatment areas with leaks, penetration problems or insufficient treatment times inevitably harbour many insects that are stressed but escape death. Resistance may also contribute to pest survival. In the laboratory, resistance development to reduced O_2 (Donahaye 1990a) and enriched CO_2 (Donahaye 1990b) has been reported. A *T. castaneum* strain that was selected for hypercarbia resistance was able to maintain water balance and energy reserves better than an unselected strain, while strains that were selected for either hypercarbia and hypoxia resistance had reduced metabolic rates (Donahaye 1992). A secondary, chemical-free control measure would be useful to address escapes and as a resistance management tactic.

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, has proven efficacy for many insect pests of stored grain and grain products but is not considered a commercially viable stand-alone option for controlling *T. castaneum* (Akbar et al. 2004; Lord 2007a). We have been investigating combination strategies as a means to improve *B. bassiana*'s performance against *T. castaneum*. Combination with diatomaceous earth was synergistic in laboratory assays (Akbar et al. 2004), and desiccation stress enhanced the fungus' efficacy (Lord 2007b). Other than toxic stress from insecticidal compounds, the effects of host stress on the performance of entomopathogenic fungi has received scant attention. Temperature and nutritional stresses

have been reported to potentiate fungus effect for some insects (Donegan and Lighthart 1989; Furlong and Groden 2003; James 2005). But effects of hypoxia and hypercarbia on fungal efficacy have not been reported. The goal of this study was to determine with maximum challenge if stresses caused by modified atmospheres would enhance *B. bassiana*'s efficacy for *T. castaneum*.

Methods

Insects and fungus

All experiments were conducted with laboratory-reared *T. castaneum* originally collected in eastern Kansas and maintained on a wheat flour diet since 1958. The *B. bassiana* was commercially produced, unformulated conidia of strain GHA (Laverlam, Butte, MT) that contained 9.4×10^{10} conidia/g. To confirm viability, the conidia were spread on Sabouraud dextrose agar (SDA) with a cotton swab and incubated for 18 h at 26°C. Germination rates were scored at 400× magnification by observing, at random, 100 conidia for the presence of germ tubes. Germination was at least 85% throughout the study.

Atmospheres

Modified atmospheres were obtained by flooding 7.5 l Pyrex desiccators with compressed nitrogen or CO_2. The gases were mixed with the aid of magnetic stir bars. Oxygen and CO_2 concentrations were measured at the beginning and end of altered atmosphere exposure with a CheckPoint gas analyser (PBI Dansensor, Glen Rock, NJ). The concentrations were selected from trial exposures to give a rate of mortality of <5%.

Assays

The gas treatments to which *T. castaneum* was exposed at the beginning of the 8-day incubation period with fungus treatments were 24, 48 or 72 h in O_2 reduced to 5 ± 1% or CO_2 increased to 40% (±2%) for larvae or 44% (±1%) for adults. Beetle larvae were used 15 days after collection of eggs following overnight oviposition. Adults were of mixed sexes and were used 7–14 days after emergence. Assay vessels were 118 ml (4 oz.) glass jars with filter paper inserted into the lid rims for gas exchange. The diluent for conidia was insect diet of crimped hard red winter wheat with 12–13% moisture. Twenty grams of wheat were introduced into each jar with 20 beetles per jar. Duplicate jars served as one replicate in order to minimize crowding. *Beauveria bassiana* concentrations were 500 mg of conidia/kg of grain for adults and 100 mg of conidia/kg for larvae. All replicates were temporal to avoid pseudoreplication from use of common cohorts and chambers. Replication was continued until there was a clear trend in the results. There were eight replicates for larvae and four replicates for adults in reduced O_2 assays. Assays with elevated CO_2 included five replicates for larvae and six replicates for adults. Relative humidity (RH) in the desiccators was adjusted to 60% with salt solution to avoid desiccation stress, and all treatments were incubated at 30°C (±1°C) in continuous darkness. Mortality was scored after 8 days. Red pigment, assumed to be oosporein, was seen in nearly all the dead larvae that had been exposed to the fungus confirming infection.

Beauveria bassiana germination and growth

To test the effects of the gas concentrations that were used in the bioassays on *B. bassiana* germination, conidia were spread on SDA in 90 mm Petri dishes and incubated under the conditions of the bioassays. For germination tests, the SDA contained 0.001% benomyl to prevent mycelial growth from obscuring conidia. Germination, as determined by the presence of a visible germ tube, was scored on 100 conidia in each of three agar plates after 24 h. For conidia that were incubated in modified atmosphere and had less germination than in ambient atmosphere controls, a second set of counts was taken after an additional 24 h in ambient atmosphere to determine whether the germination reduction was due to delay or death. For growth measurement, 90 mm SDA plates without benomyl were inoculated with 3 mm cores cut from SDA plates that had been inoculated with ca. 10^7 conidia/ml 24 h earlier. Three replicate plates were incubated in 5% O_2 or 40% CO_2 at 30°C for 24, 48 and 72 h with ambient atmosphere control for each. Growth was assessed by measuring across the widest diameter at the end of each period. Both experiments were carried out three times. There were no statistically significant differences among the results of the iterations, and the data were pooled.

Statistics

Data were subjected to ANOVA with SigmaStat 3.1 (Systat, Point Richmond, CA). Raw data that did not pass the Kolmogorov–Smirnov test for normality (pupation and larval mortality with reduced O_2)

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were transformed to arcsine square roots. Differences among means were detected with Tukey's test. Mortality of fungus treated beetles was adjusted with Abbott's (1925) formula for mortality of control insects that were exposed to the same atmospheric conditions. Pupation data was subjected to two-way ANOVA. When the effect of reduced O_2 on pupation of survivors that were not exposed to *B. bassiana* was analysed, variances were unequal and the Kruskal–Wallis test was used.

Results

Larval mortality

The efficacy of *B. bassiana* for control of larval *T. castaneum* was greater with O_2 reduction to 5% (±1%) for 72 h than in ambient air ($F_{3,28} = 5.5$, P = 0.004), but 24 or 48 h of reduced O_2 did not have a significant effect (fig. 1). Exposure of larvae to 40% (±2%) carbon dioxide did not affect the mortality of *B. bassiana*-exposed larvae ($F_{3,16} = 0.3$, P = 0.82; fig. 2). Interactions of fungus treatment with exposure to both reduced O_2 ($F_{3,48} = 0.02$, P = 0.99) and elevated CO_2 ($F_{3,32} = 0.21$, P = 0.89) were not significant.

Adult mortality

Mortality of *B. bassiana*-exposed adult beetles was significantly greater with exposure to 44 ± 1% carbon dioxide for 72 h compared with exposure to 0, 24 or 48 h ($F_{3,20} = 4.1$, P < 0.05; fig. 3). There was no significant effect on adult mortality of O_2 reduction to 5% (±1%) ($F_{3,12} = 0.2$, P = 0.89), and the mortality with or without exposure ranged only from 9% to 12% (data not shown).

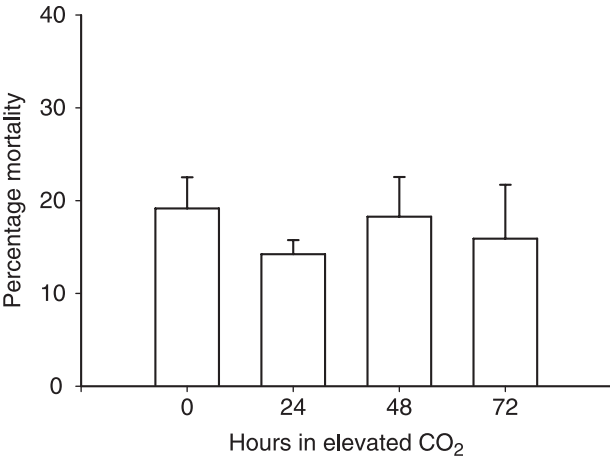

Fig. 2 Abbott-corrected mortality (SE) of *Tribolium castaneum* larvae exposed to *Beauveria bassiana* with elevated CO_2 (40 ± 2%) and 8 days of incubation (n = 5).

Pupation

Pupation of larvae that were treated 15 days after oviposition was significantly affected by O_2 reduction ($F_{3,48} = 25.5$, P < 0.01) and by CO_2 elevation ($F_{3,39} = 5.1$, P = 0.006) but not by the presence of *B. bassiana* in either the experiments with reduced O_2 ($F_{3,48} = 3.1$, P = 0.09) or elevated CO_2

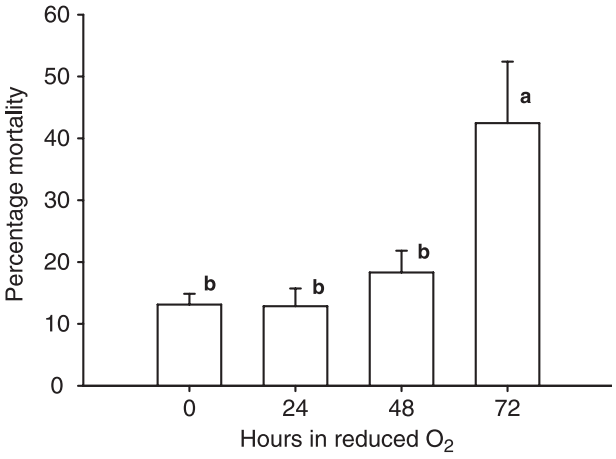

Fig. 1 Abbott-corrected mortality (SE) of *Tribolium castaneum* larvae exposed to *Beauveria bassiana* with oxygen reduction (5 ± 1%) and 8 days of incubation (n = 8). Bars with the same letter are not significantly different (α = 0.05).

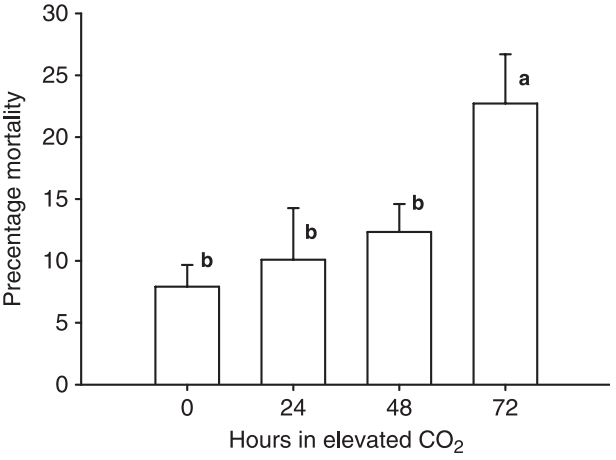

Fig. 3 Abbott-corrected mortality (SE) of *Tribolium castaneum* adults exposed to *Beauveria bassiana* with elevated CO_2 (40 ± 2%) and 8 days of incubation (n = 4). Bars with the same letter are not significantly different (α = 0.05).

Table 1 Effect of reduced oxygen, elevated carbon dioxide and exposure to *Beauveria bassiana* on *Tribolium castaneum* percentage pupation (±SE)[1]

	Hours in altered atmosphere			
	0	24	48	72
Reduced O_2				
Beauveria bassiana	87.9 ± 2.1 a	60.7 ± 7.3 ab	37.9 ± 9.1 bc	20.7 ± 8.8 bc
Control	94.6 ± 2.1 a	70.4 ± 4.8 ab	47.5 ± 10.1 b	32.5 ± 10.9 b
Elevated CO_2				
Beauveria bassiana	80.0 ± 8.1 a	63.5 ± 15.1 ab	46.5 ± 11.7 ab	46.0 ± 11.1 b
Control	93.5 ± 5.3 a	72.5 ± 14.1 ab	66.5 ± 13.7 ab	55.0 ± 11.1 b

[1]Mean values within rows followed by the same letter are not significantly different ($\alpha = 0.05$).

($F_{3,39} = 3.5$, P = 0.07) (table 1). Both interactions of fungus treatment with exposure to reduced O_2 ($F_{3,48} = 0.02$, P = 0.99) and elevated CO_2 ($F_{3,32} = 0.21$, P = 0.89) did not significantly affect pupation. Pupation of survivors that were not exposed to *B. bassiana* was significantly affected by reduced O_2 (P < 0.01) and elevated CO_2 ($F_{3,82} = 3.82$, P = 0.03).

Beauveria bassiana germination and growth

After 24 h of incubation on agar, germination of *B. bassiana* conidia was significantly lower in elevated CO_2 than in ambient atmosphere ($F_{2,24} = 6.2$, P = 0.006). After an additional 24 h, germination of the conidia that were incubated in enriched CO_2 did not differ from the control. Oxygen displacement did not affect germination significantly ($F_{2,23} = 1.2$, P = 0.31) (table 2).

Both atmosphere modifications slowed mycelial growth (table 3). Growth from inoculation disks with reduced O_2 was significantly less than in ambient atmosphere at both 24 h ($t_{16} = 2.6$, P = 0.02) and 48 h ($t_{16} = 3.1$, P = 0.006). Similarly, growth from inoculation disks under elevated CO_2 was significantly less than in ambient atmosphere at both 24 h ($t_{16} = 8.8$, P < 0.001) and 48 h ($t_{16} = 15.0$, P < 0.001).

Table 2 *Beauveria bassiana* germination (±SD) under modified atmospheres

Hours in MA	Germination (% ±SD)
5% (±1%) O_2	
0	86.7 (±3.8) a
24	83.5 (±4.9) a
40% (±2%) CO_2	
0	87.1 (±3.3) a
24	74.6 (±11.3) b
48	82.4 (±5.9) a

[1]Mean values for a given atmosphere modification followed by the same letter are not significantly different ($\alpha = 0.05$).
MA, modified atmospheres.

Table 3 *Beauveria bassiana* mycelial growth (mm ± SD) with modified atmospheres[1]

	5% (±1%) O_2		40% (±2%) CO_2	
	Control	Treated	Control	Treated
24 h	0.67 (±0.21)	0.36 (±0.28)	0.60 (±0.11)	0.26 (±0.04)
48 h	1.84 (±0.33)	1.39 (±0.27)	1.83 (±0.19)	0.61 (±0.16)

[1]All the differences between paired treatment and control mean values were significant ($\alpha = 0.05$).

Discussion

The O_2 and CO_2 concentrations that were used in this study induce similar stresses on the beetles, and both caused <5% mortality. Yet, the effect of O_2 reduction for 72 h was significant for larvae while the effect of CO_2 elevation was not. This may be explained, at least in part, by their effects on the fungus. The O_2 reduction had no effect on germination and a small but significant effect on growth, while CO_2 elevation reduced both germination and mycelial growth. The reduced rate of hyphal growth that was observed with modified atmosphere would be operative only in the interval between germination and host penetration. Given that the reduced rates of growth on agar in this study were 10.8 μm/h in hypercarbia and 15.0 μm/h in hypoxia, as compared with ca. 26 μm/h in control atmosphere, and that the hyphae grow only a few μm on the insects' cuticles before penetration, there is probably <1 h of cuticular growth involved. Only if the insect molted during that period would infection rates be affected. On the other hand, the reduction in germination rate probably has more effect, as it is equivalent to loss of inoculum. Perhaps, the CO_2 inhibition of the

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fungus acted as a countervailing factor to the beetle stress that resulted in no significant change in *B. bassiana* effect on beetle larvae.

In an apparent anomaly in this study, reduced O_2 resulted in a significant increase in *B. bassiana*-associated mortality of larvae mortality but increased CO_2 did not, while for the adults, this was reversed, with elevated CO_2 but not reduced O_2 having a significant effect on fungus-associated mortality. A partial explanation for this can be found in the difference in effects of the two atmospheres on pupation. Oxygen reduction had a stronger effect on the rate of larval development to pupation than did CO_2 increase. Accordingly, the intermolt period of the host larvae was greater in reduced O_2, and there would be less loss of inoculum on cast exuvia. An explanation for the adult responses is more elusive. Adult *T. castaneum* are less susceptible to *B. bassiana* than the larvae are, and in a previous study we were unable to obtain a dose-mortality response (Akbar et al. 2004). There are great differences between the larval and adult stages. Importantly, the cuticles are very different. Perhaps, a difference in response is to be expected.

In these assays, CO_2 was introduced by displacement of ambient gases. This results in a reduction of oxygen to 12% concomitant with a CO_2 increase to 40%, and that may contribute to any effect. As the oxygen reduction was carried out by nitrogen injection, no other gas with the possible exception of water vapour can be implicated. Modified atmospheres act in concert with moisture. In addition, oxygen deprivation and exposure to elevated CO_2 induce opening of spiracles for longer than normal periods. Pearman and Jay (1968) reported that mortality of larval *T. castaneum* that were exposed to elevated CO_2 was greatest at low humidities. Jay et al. (1971) reported similar results with adult *T. castaneum*, *T. confusum* Jacquelin du Val and *Oryzaephilus surinamensis* (L) exposed to reduced O_2 or elevated CO_2. In the assays in this study, the RH was maintained at ca. 60% (1.69 kPa vapour pressure deficit). Under normal atmospheric gas concentrations, that would not cause desiccation stress, but with extended spiracle opening it may have been a factor. The author has previously reported that mortality of *B. bassiana*-treated *T. castaneum* increases with decreasing moisture (Lord 2007a). While maintaining the incubation conditions at 60% RH kept desiccation effects low, a possible role in the gas effect cannot be ruled out.

There are many other factors that might be implicated in increasing pathogen efficacy under modified atmospheres. The stresses that were imposed on the beetles slowed the development of the larvae as was indicated by the reduction of pupation with altered atmosphere. This creates longer intermolt periods that may favour fungal attack by reducing the loss of inoculum with shed exuvia. The physiological effects of modified atmospheres are very complex and include weight loss, changes in carbohydrate, lipid and protein metabolism and abundance, and acidosis in the case of CO_2 enrichment (Jay and Cuff 1981; Nicolas and Sillans 1989; Mitcham et al. 2006). Biron et al. (2005) reported that several proteins that were induced by infection with microsporidia, which are specialized fungi, in the mosquito, *Aedes aegypti* (L), were suppressed by hypoxia, suggesting possible immune suppression. Determination of the relative importance of these factors would allow their direct exploitation.

One of the questions that provided impetus for this study was whether adult secretions would have an impact on fungal efficacy. Adults of *T. castaneum* produce defensive compounds that are not present in the larvae. One of the documented effects of CO_2 but not N_2 at concentrations that do not arrest activity is an increase in the secretion of *p*-benzoquinones (Irwin et al. 1972). Reduced oxygen does not induce secretion of *p*-benzoquinones. As these are defensive secretions with anti-fungal activity (Prendeville and Stevens 2002), that effect would be detrimental to *B. bassiana* efficacy. As elevated CO_2 had a significant effect on the mortality of *T. castaneum* that were exposed to *B. bassiana* but reduced oxygen did not, it appears that the quinones are not effective defenses against the fungus.

There was only one concentration used for each gas in this study. They were chosen to cause <5% mortality. Preliminary tests showed that a very slight increase in CO_2 concentration or a very slight decrease in O_2 concentration from those that were used caused 100% mortality. Accordingly, these results show near maximum effect of the oxygen and CO_2 stress on *B. bassiana* efficacy for the red flour beetle.

There are many situations in which O_2/CO_2 modification might increase fungal efficacy where there is survival of stressed insects. With atmosphere modification presently being in use in stored products, it is among the most apparent. But modifying atmosphere may require several days of purging and may result in unacceptable pest survivorship. Hermetically sealed containers have long been seen as an effective chemical-free method of protecting stored commodities from insects. But, depending on factors

including temperature, population and commodity, the time for gases to reach lethal concentrations can be very long (Press and Harein 1967b; Aliniazee 1971). *Beauveria bassiana* can be a supplemental control agent where chemical use is not desirable, but the results of this study indicate that enhancement of the fungus' efficacy with modified atmosphere would be modest and require at least 3 days of continued hypoxia or hypercarbia. The concentrations and combinations of gases in modified atmospheres are limitless. Field trials would be needed to assess the practicality of this approach.

The mortality in these assays is well below what is practical for pest control. One reason for this is that the author chose *T. castaneum* as the target because it is not only a major pest of stored products but also one the most tolerant of entomopathogenic fungi. It is therefore a target for which the efficacy of *B. bassiana* needs to be improved. Another reason for the low mortality is that the fungus doses and gas modifications were chosen to cause low mortality in order to maximize the detectability positive interactions. Higher doses and less tolerant target insects may give more promising results.

In summary, O_2 reduction and CO_2 increase can increase *B. bassiana*'s efficacy for *T. castaneum*, one of the most fungus-tolerant stored-product pest insects. The reduction in beetle development rate is one factor that would favour the increase. On the other hand, both gas modifications reduce the fungus' growth rate, and CO_2 increase slowed the germination of conidia. Apparently, the stress to the insect is a more important determinant than the stress on the fungus.

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